Influence of substituents on the solution conformation of the exopolysaccharide produced by *Pseudomonas* 'gingeri' strain Pf9

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Abstract

The influence of pyruvate ketals and acetyl groups on the conformational behaviour of the exopolysaccharide produced by *Pseudomonas* 'gingeri' strain Pf9 has been investigated experimentally through studies of intrinsic viscosity and circular dichroism experiments. A conformational variation was detected as a function of the ionic strength. Measurements carried out on the native polymer, as well as on both de-pyruvated and de-acetylated samples, suggested a critical role for the acetyl group on the solution conformation of the polysaccharide. Molecular mechanics calculations indicated the possibility of intramolecular hydrogen bonding between acetyl substituents on the mannose and the C(2)OH group of the preceding saccharidic unit. NMR linewidth measurements, carried out as a function of temperature, on the low molecular weight de-pyruvated sample indicated different polymeric backbone dynamics in aqueous solutions with respect to that observed in 0.3 M NaCl solutions.

Keywords: Microbial polysaccharides; *Pseudomonas*; Structure–function relationships; Conformational properties; Acetyl and pyruvate groups

1. Introduction

Exopolysaccharides produced by different *Pseudomonas* species have been investigated in connection with bacterial diseases of cultivated mushrooms [1]. Recently, the primary structure of the exopolysaccharide produced by *Pseudomonas* 'gingeri' strain Pf9 was reported [2,3]. This bacterium produces ginger-coloured lesions on the cap of the commercial mushroom *Agaricus bisporus*. The chemical structure of the exopolysaccharide (hereafter referred as *ExoPf9*, Fig. 1) is unusual due to the substitution of the mannose residues with both pyruvic ketal groups and acetyl substituents [3]. It is worth noting that *ExoPf9* has the same structure as the *Escherichia coli* K55 capsular polysaccharide [4] and differs from that of the *Klebsiella pneumoniae* K5 capsular polysaccharide only in the position of acetylation [5]. Due to the presence of pyruvic ketal groups and acetyl substituents on the mannose residues all the hydroxyl groups of this sugar are unavailable for further interactions in the polymer leading to a sterically hindered structure.

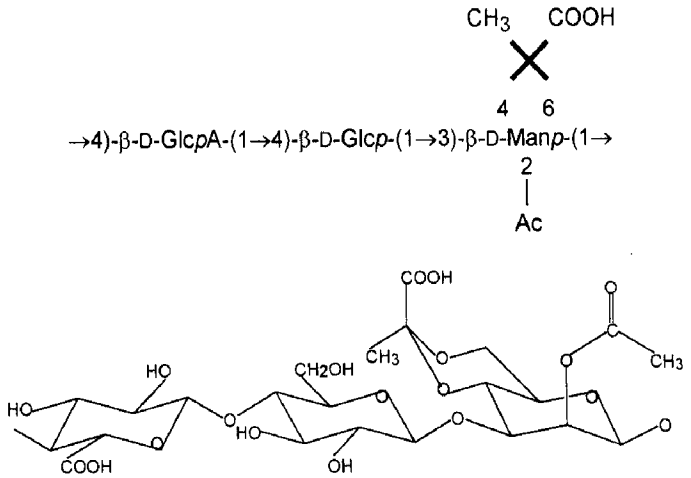

Fig. 1. Chemical repeating unit structure of the exopolysaccharide produced by *Pseudomonas* 'gingeri' strain Pf9.

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In order to explore the influence of such substituents on the conformational behaviour of the polysaccharidic chain, both the chiro-optical behaviour and the viscosity properties of *ExoPf9* were studied as a function of ionic strength and temperature. The investigation was carried out on the native polymer as well as on de-pyruvated and de-acetylated samples.

2. Experimental

The exopolysaccharide was obtained from cultures of *Pseudomonas* 'gingeri' strain Pf9 (ATCC 51311) as described by Fett et al. [2]. The polysaccharide was further purified by treatment with DNAse, RNAse and protease (Sigma) followed by extensive dialysis. Molecular weight measurements were carried out by means of high pressure size exclusion chromatography coupled with low angle laser light scattering detection, using a set of three Supelco TSK-PWXL columns (G-6000, G-5000, G-3000) in series.

De-pyruvylation was carried out by heating a polysaccharide solution at 100°C for 2.5 h in the presence of 50 mM oxalic acid [6]. After neutralisation, the resultant solution was dialysed against 0.1 M NaCl and de-ionised water. The de-acetylated sample was obtained by treating with base (10 mM NaOH) under a nitrogen flux for 5 h at room temperature. After neutralisation, the resultant solution was dialysed against 0.1 M NaCl and de-ionised water. The samples were lyophilized and stored at 5°C.

Capillary viscosity measurements were carried out using an automatic apparatus (Schott-Geräte AVS 440) provided with an Ubbelohde (0c) capillary viscometer at controlled temperature (25°C). The capillary diameter was 0.46 mm and the relative viscosity was in the range 1.32–1.99; the flow rate of the 0.1 M NaCl aqueous solvent was 315.4 s.

Circular dichroism spectra were made on a Jasco J-600 instrument provided with temperature control.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC200 spectrometer operating at 4.7 T.

Molecular mechanics calculations were performed by means of in-house software [7]. Refinement of the low energy structure of the β-D-Glc*p*-(1-3)-β-D-Man*p* dimer was obtained by means of NMR-Graph (version 3.1) software package (NMR-graph, Molecular Simulation, Burlington, MA, 1991) which is provided with Dreiding II force field [8].

3. Results and discussion

The chemical characterisation of the native polymer, as well as that of pyruvate-free and acetyl-free derivatives, was performed by means of ^{1}H- and ^{13}C-NMR spectroscopy. The integrated area of the ^{1}H-NMR peaks relative to pyruvate and acetyl groups (1.47 and 2.14 ppm, respectively) gave a molar ratio equal to 1:0.6, revealing that not all the mannose units carried both substituents. In fact, the degree of acetylation may depend either on the cultivation conditions, or on the stability of the strain, since in different polysaccharide preparations [3] the molar ratio was found to be 1:1.

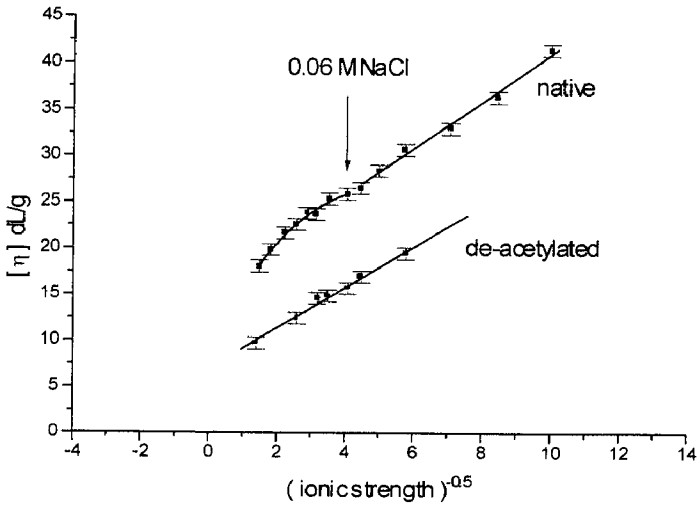

Fig. 2. Intrinsic viscosity, reported as a function of the inverse of the square root of the ionic strength, for the native *ExoPf9* and the de-acetylated sample.

For pyruvate-free derivatives, the ^{1}H-NMR spectrum showed that, after 2.5 h of acidic treatment, 4% of the pyruvic acetals were still present on the polymer. The time of the treatment was not prolonged in order to avoid further polymer degradation. The weight average molecular weight of native *ExoPf9* was 1.27×10^{6} g/mol, in agreement with published results [9], whereas that of the pyruvate-free polymer was 3.7×10^{4} g/mol. Similarly, treatment with base for the displacement of acetyl esters resulted in a decrease of the molecular weight (7.23×10^{5} g/mol). However, the removal of the substituents was complete as indicated by the ^{1}H-NMR spectrum (data not shown).

The behaviour of *ExoPf9* was investigated by dilute solution capillary viscometry. Due to the ionic nature of the polymer (Fig. 1), the intrinsic viscosity was measured as a function of the inverse of the square root of the ionic strength [10], and the experimental data are shown in Fig. 2. The data indicated the presence of rather viscous systems that are typical of carbohydrate polymers exhibiting high molecular weight and some conformational order. The $[\eta]$ value at 0.1 M NaCl was equal to 25 dl/g, in excellent agreement with the value obtained using a different experimental apparatus [9]. For the sake of comparison, similar values were obtained in the case of the gelling algal polysaccharide alginate in the sodium salt form [11]. The plot (Fig. 2) showed the usual decrease of $[\eta]$ for increasing values

of ionic strength (I), but was not linear, as expected for polyelectrolytes in the absence of conformational transitions. A deviation from linearity was found for I values equal to 0.06, where the $[\eta]$ vs. $I^{-0.5}$ curve exhibited a change in slope. Since this change was small, further experiments were performed to confirm these data, looking for reproducibility on one hand, and resorting to the use of different techniques on the other hand. The first objective was easily resolved as indicated in Fig. 2 where the experimental errors are reported. Amongst the alternative techniques available, circular dichroism (CD) spectroscopy was chosen to test for possible conformational changes and experiments were carried out on *ExoPf9* as a function of ionic strength. As shown in Fig. 3, the native polymer exhibited a variation in the molar ellipticity of the chromophore groups at an I value of around 0.06, where the deviation from linearity in the $[\eta]$ plot occurred. Therefore, both intrinsic viscosity and circular dichroism data strongly suggested that the screening of the negative charges on the polysaccharidic chain promoted a transition towards a more rigid conformation.

Molecular mechanics calculations, performed on the repeating unit of *ExoPf9*, showed the possibility for the occurrence of an efficient hydrogen bond between the acetyl group at the 2-position of the mannose residue and the hydroxyl group at the 2-position of the preceding glucose residue (Fig. 4a). The formation of this hydrogen bond could be induced after the ionic strength screened part of the negative charges, thus rendering accessible a suitable local conformation. However, the degree of acetylation on the polymer being only about 60%, hydrogen bonding along the polymeric chain would remain largely incomplete, thus explaining the limited extent of the increment observed for the native *ExoPf9* intrinsic viscosity.

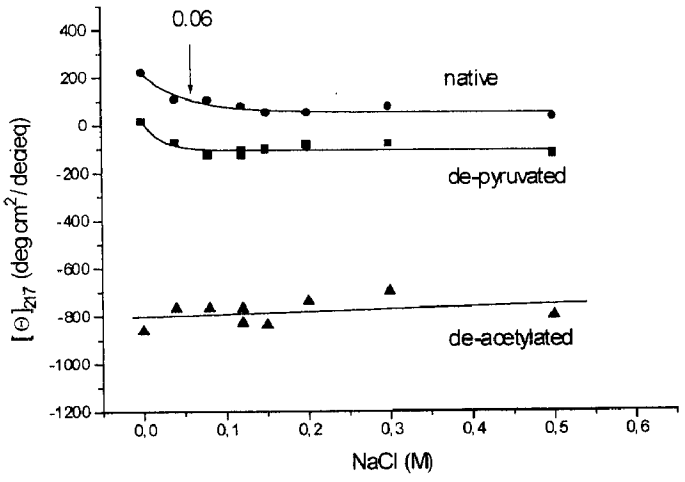

Fig. 3. Molar ellipticity as a function of the ionic strength for native, de-pyruvated and de-acetylated samples.

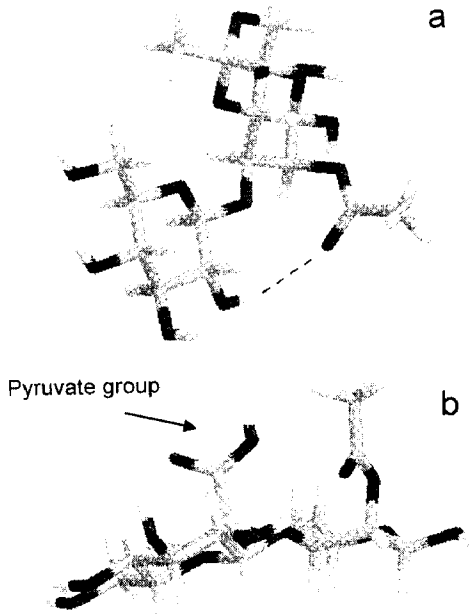

Fig. 4. Structure of the β-D-Glc*p*-(1-3)-β-D-Man*p* dimer in the energy minimum conformation exhibiting: (a) the hydrogen bond between the mannose acetyl substituent and the glucose C(2)OH group and (b) the position of the pyruvate carboxylate group.

The behaviour of the molar ellipticity at 217 nm for the de-pyruvated sample as a function of the ionic strength is shown in Fig. 3. As can be seen, the shape of the curve was very similar to that obtained for the native polymer except for a general shift of $[\Theta]$ to lower values due to the absence of the contribution of the pyruvic carboxylate chromophores. The $[\eta]$ vs. $I^{-0.5}$ plots for the de-pyruvated polymers was not obtained: its comparison with the equivalent plot of the native polymer would be difficult anyhow because of the difference in charge density and molecular weight exhibited by the two polysaccharidic samples. The comparison of the CD spectra of the native and the de-pyruvated sample strongly suggested that the two polymers exhibited very similar behaviour in the presence of increasing ionic strength.

Both CD and intrinsic viscosity measurements of the de-acetylated sample carried out as a function of the ionic strength showed different behaviour. In fact, the $[\Theta]_{217}$ values stayed constant over the 0–0.5 M range of NaCl concentration (Fig. 3). The plot of $[\eta]$ vs. $I^{-0.5}$ for the de-acetylated sample is also shown in Fig. 2. In this case, the behaviour is linear over the entire ionic strength range investigated. In addition to this, the slope of the curve normalised for the value of the intrinsic viscosity at 0.1 M ionic strength (B value, as described by Smidsröd and Haug [8]) indicated a higher flexibility of the de-acetylated polymer with respect to the native one (native: $B = 0.055$; de-acetylated: $B = 0.062$). In the case of native *ExoPf9*, the slope of the

plot of $[\eta]$ vs. $I^{-0.5}$ was evaluated in the range 5–10 of $I^{-0.5}$ to avoid ionic strength values where the plot was not linear.

Furthermore, CD experiments carried out in water as a function of temperature showed conformational differences between de-acetylated, on one hand, and both native and de-pyruvated polymers on the other hand. As shown in Fig. 5, both native and de-pyruvated samples exhibited a negative slope, probably due to an increasing rotational mobility of acetyl chromophore groups, which was more apparent for the de-pyruvated molecules. Contrary to this, the de-acetylated sample displayed a constant behaviour.

The experimental data described above indicated that the acetyl groups present on the 2-position of the mannose units were crucial for the definition of the conformational behaviour of *ExoPf9* in aqueous solutions. The possible formation of a hydrogen bond between acetyl groups and the hydroxyl groups placed at the 2-position of the preceding 3-linked glucose residue could reasonably explain the above conclusion. In addition to this, the conformation stabilised by this hydrogen bond had the pyruvate carboxylate group directed towards the solvent (Fig. 4b) almost perpendicular to the plane of the saccharidic ring and unable to intramolecularly interact with other neighbouring saccharidic units. Therefore, these structural characteristics may well explain why the removal of the pyruvate group had little effect on the conformational behaviour of *ExoPf9*.

As mentioned before, the de-pyruvation procedure caused a decrease of the molecular weight of the polymer. Although this degradation did not allow a comparison of the intrinsic viscosity data with those relative to the native polymer, it was exploited to carry out ^{1}H-NMR linewidth experiments. In fact, the lowered viscosity of the de-pyruvated sample solutions allowed reasonably well resolved proton NMR spectra to be

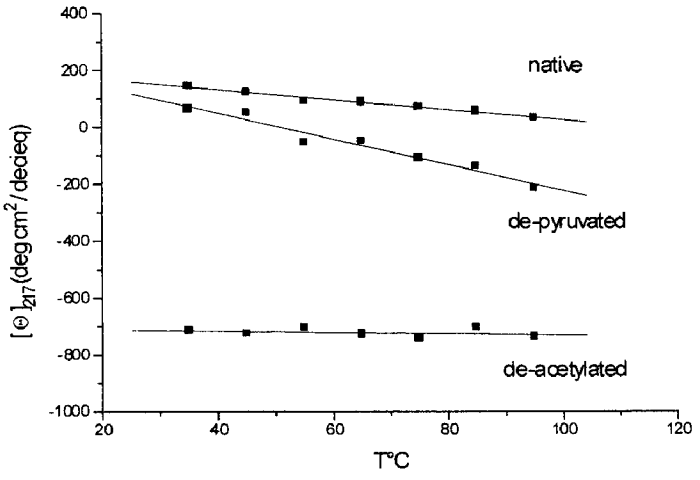

Fig. 5. Molar ellipticity as a function of temperature for native, de-pyruvated and de-acetylated samples.

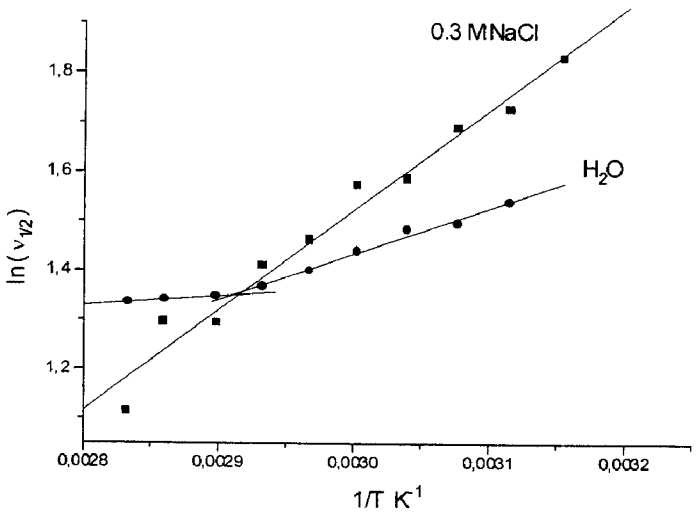

Fig. 6. Plot of the logarithm of the mannose anomeric-proton linewidth as a function of $1/T$ for de-pyruvated sample in water and 0.3 M NaCl.

obtained. In particular, the signal relative to the anomeric proton of the acetylated mannose unit (4.84 ppm), and that relative to the H-2 proton of the same residue (5.52 ppm), were well resolved allowing careful linewidth ($\nu_{1/2}$) measurements.

As reported by Lane et al. [12], a plot of $\ln(\nu_{1/2})$ as a function of $1/T$ is linear for macromolecules, since the terms relative to the high frequency component of the spectral density function, $J(\omega)$, are small in comparison to the term of the fundamental component, $J(0)$. In addition to this, the slope of the plot can be correlated with the activation energy of the molecular motions, both tumbling and segmental, the former being dominant for polymeric molecules.

In the case of de-pyruvated *ExoPf9*, $\ln(\nu_{1/2})$ values for the NMR peaks of the mannose anomeric proton were measured both in water and in 0.3 M NaCl within a temperature range from 40 to 85°C. The experimental findings are shown in Fig. 6.

As can be seen, the slope of the curve obtained in 0.3 M NaCl was slightly higher than that obtained in water. Therefore, the energy required to increase the molecular motions of the molecules was higher in 0.3 M NaCl solution than in water. These findings agreed well with the circular dichroism data that indicated, for the de-pyruvated sample at high ionic strength, a conformation very similar to that of the native polymer, probably characterised by inter-residue hydrogen bonding along the polymeric chain. In addition to this, the plot of $\ln(\nu_{1/2})$ vs. $1/T$ in water exhibited a peculiar trend. In fact, a discontinuity in the slope was observed at around $T = 70$°C where the slope, and thereafter the motional activation energy, decreased markedly. The same discontinuity was not observed by means of circular dichroism experiments carried out in water as a

function of temperature (Fig. 5; de-pyruvated sample). This discrepancy in the results obtained may be explained by assuming that the discontinuity was mainly caused by intermolecular interactions which induced restrictions in the tumbling of the polymeric molecules. An increase of the temperature may lead to a decrease of the excluded volume size thus explaining the behaviour shown in Fig. 6. The phenomenon may, therefore, be considered very similar to a transition from solution behaviour in the dilute regime to that in the semi-dilute one.

4. Conclusions

Microbial exopolysaccharides provide a viscous aqueous environment surrounding bacteria, which is believed to help the biological functions of the microorganisms. Similar to other exopolysaccharides, the viscosity of aqueous solutions of the polymer produced by *Pseudomonas* 'gingeri' strain Pf9 was rather high. For the sake of comparison, the same intrinsic viscosity ($[\eta] = 25$ dl/g at 0.1 M ionic strength) was obtained for a sodium alginate sample exhibiting a molecular weight of 1×10^6 g/mol [11]. In addition to this, the *B* rigidity factor [11] suggested that the exopolysaccharide was slightly more flexible than alginate. Dilute solution capillary viscometry, circular dichroism and NMR spectroscopy suggested that the conformational behaviour of the exopolysaccharide is influenced by the presence of an acetyl substituent on the mannose residue through possible hydrogen bonding with the preceding glucose residue. The complete removal of the acetyl groups led to a drop in the value of the intrinsic viscosity, measured at 0.1 M ionic strength, down to 10 dl/g.

It may be postulated that the control of the flexibility, and therefore the control of the viscosity of the bacterial extracellular medium, is determined by the degree of esterification which can vary for different cultivation conditions up to a 1:1 molar ratio between pyruvic acetal and acetyl groups [3].

Acknowledgements

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